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THE EFFECTS OF ACTH, ISOPROTERENOL AND DEXAMETHASONE ON THE RAT ADRENAL GLAND RESPONSE TO ETHANE DIMETHANESULPHONATE (EDS): A STEREOLOGICAL STUDY

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Ethane dimethanesulphonate (EDS), an alkylating agent, caused marked atrophy of the adrenal cortex of adult male rats, in addition to its toxic effect on testicular Leydig cells. The aim of this work was to examine whether a 9-day treatment with ACTH (40 IU/kg/d), isoproterenol (120 μg/kg/d) or dexamethasone (0.25 mg/kg/d), which started 4 days prior to intraperitoneal administration of a single dose of EDS (75 mg/kg), affected the morphological changes in the adrenal cortex evoked by EDS alone. The animals were killed 15 days after EDS injection. Stereological analysis revealed that both ACTH and isoproterenol almost completely prevented cortical atrophy induced by EDS. They also considerably stimulated corticosterone secretion in EDS-injected animals. By contrast, in dexamethasone-suppressed rats, the deleterious effect of EDS on adrenocortical cells was augmented. The volume and cellularity of all cortical zones were reduced, but the remaining cells of the zona reticularis displayed considerable hypertrophy, which was probably responsible for the maintenance of corticosterone secretion. These results clearly demonstrate that both ACTH and β adrenoceptor stimulation have protective action against the toxic effects of EDS on rat adrenal cortex, whereas dexamethasone exerts an opposite influence.

Key words: Ethane dimethanesulphonate, rat adrenal cortex, ACTH, isoproterenol, dexamethasone, stereology, corticosterone.

INTRODUCTION

The methane sulphonates are alkylating agents, which have been developed for chemotherapy and are often used in examining genotoxicity (Shealy and Krauth, 1993; Ehling and Neuhäuser-Klaus, 1995). Originally, ethane 1,2-dimethane sulphonate (EDS) was considered to destroy Leydig cells specifically in the testis of adult rats and some other rodents, leading to very low levels of testosterone in the blood and transient infertility (Jackson and Jackson, 1984; Klinefelter *et al*., 1991). However, increasing data indicate that EDS may have more extended effects that are independent of the elimination of Leydig cells. Thus, the agent has

been shown to influence directly the rat epididymal epithelium (Klinefelter *et al*., 1992), the seminiferous epithelium (Sprando *et al*., 1990), adenohypophysial gonadotropes (Dong and Handelsman, 1991) and thymocytes (Morris *et al*., 1997). We have previously reported that EDS has a strong deleterious effect on steroidogenic cells of adult male rat adrenal cortex (Plećaš *et al*., 1997), which was manifested by marked atrophy of the inner cortical zone 15 days after administration of EDS. Our further investigation showed that the secretory response of adrenocortical cells to EDS can be modified in an opposite fashion with ACTH and dexamethasone (Plećaš *et al*., 2001).

The aim of this work was to examine the morphometric characteristics of rat adrenal glands that, at the time of EDS application, were stimulated by exogenous ACTH or suppressed by dexamethasone. Besides that, since we have reported that chronic administration of β-adrenergic receptor agonist or antagonist might influence the structure (Plećaš *et al*., 1996) and function (Plećaš *et al*., 1999) of the adrenal cortex, the effects of EDS were also examined in animals treated with the β-adrenergic receptor agonist isoproterenol.

MATERIAL AND METHODS

Adult male Wistar rats, weighing 250-300 g at the beginning of the experiment, were housed under conditions of controlled temperature (19-21°C) and light (12 h darkness/12 h light) with constant access to a standard diet and tap water. They were divided into groups (n=7 each) that were injected subcutaneously with saline (1 ml/kg/d), ACTH (40 IU/kg/d; ACTH, ICN-Galenika), isoproterenol (120 μg/kg/d; Isoproterenol hydrochloride, SIGMA) or dexamethasone (0.25 mg/kg/d; Dexamethasone, ICN-Galenika) for 9 consecutive days. On day 4 of the treatment, all the animals received a single intraperitoneal injection of EDS (75 mg/kg). An additional control group was injected with saline for 9 days, but on the 4th day received intraperitoneally the vehicle for EDS, dimethylsulphoxide (DMSO) in water (3:1 v/v). Since EDS is not commercially available, it was synthesised in our laboratory by the procedure described by Jackson and Jackson (1984). The rats were killed 15 days following EDS or DMSO administration.

To avoid corticosterone fluctuations due to the circadian rhythm, all experiments were performed between 9-11 h. The animals were handled gently and all efforts to minimise stress were made.

At the time of killing, rats were anaesthetised with ether and blood samples were taken by cardiac puncture within 2 min of total anaesthesia (Villas *et al*., 1991). After decapitation, the adrenal glands were dissected out and individually weighed.

Morphometric analysis

The left adrenal gland was fixed in Bouinžs fluid, dehydrated in a graded series of alcohols and embedded in paraffin. Serially cut sections, 6 μm thick, were stained with hematoxylin and eosin.

Stereological measurements were carried out by a procedure similar to that described by Malendowicz (1997) as reported earlier (Plećaš *et al*., 1990).

Level I: Zonation of the adrenal gland. In order to evaluate the volumetric densities of individual adrenocortical zones (*zona glomerulosa*, ZG; *zona fasciculata*, ZF; *zona reticularis,* ZR) and of the adrenal medulla, every sixth section of the gland was measured by a point counting method at a 125 x magnification and with a square-lattice test system of type A (Weibel, 1979). The volume of the fixed gland was also determined stereologically, by counting the number of points falling on the gland on every sixth section.

Level II: *Size and number of adrenocortical cells.* One or two equatorial sections of each gland were chosen and 30 test areas of ZG and 50 test areas of ZF and of ZR were estimated at a 1000 x magnification with the multipurpose test system M_{42} (Weibel, 1979). From the volume fractions of nuclei and cytoplasm, as well as the number of nuclear profiles of parenchymal cortical cells, the average volume of adrenocortical cells and their nuclei and the number of adrenocortical cells in each zone were calculated.

Biochemical determinations

The right adrenal gland was immediately frozen and kept at -70 ^{o}C until corticosterone determination by high performance liquid chromatography (HPLC). Adrenal corticosterone levels were expressed as ng *per* mg of adrenal tissue.

After separation, the serum was frozen and stored at –20 ^{0}C until assayed. Serum levels of corticosterone were estimated by RIA with a commercially available kit for rats ([^{125}I]corticosterone radioimmunoassay, ICN Biomedicals, CA).

Statistics

The results are presented as means±SEM. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by the least significant difference test (LSD). $p<0.05$ was considered statistically significant.

RESULTS

The results are shown in Table 1 and Figure 1.

Fifteen days following the administration of a single dose of EDS to saline-injected rats marked changes were found in the stereological parameters of the adrenal gland comparing to the values obtained in saline and DMSO-injected controls. Significant reductions were observed in the volumes of the gland, cortical zones and the medulla. EDS reduced the number of parenchymal cells in all three cortical zones. In the ZG and ZF the mean cell volume was decreased, whereas the mean nuclear volume was decreased in the ZF and ZR. Despite these prominent morphological changes in the gland, adrenal and serum corticosterone concentrations were not significantly altered.

In the rats which on the 4th day of the 9-day ACTH treatment received EDS, adrenal volume was greater than in animals injected with saline and EDS and was similar to the value found in saline and DMSO-injected controls. The volumes of the ZG and ZF were increased. In the ZG only the cell volume was increased, whereas in the ZF enhancements were found both in the cellular and nuclear volumes. The mean cellular volume was increased in the ZR, as well. Corticosterone

Table 1. The effects of ACTH, isoproterenol and dexamethasone on stereological parameters of adrenal glands of adult male rats exposed to a single injection of ethane dimethanesulphonate (EDS)

	Saline + DMSO-vehicle	Saline + EDS	ACTH + EDS	Isoproterenol + EDS	Dexamethasone + EDS
Absolute volume (mm^3)					
Right adrenal gland	13.70±1.11	9.01±0.74#	14.41±0.63*	14.41±0.97*	4.21±0.33*
ZG	2.108±0.251	1.563±0.118#	2.030±0.110*	1.959±0.124	0.909±0.044*
ZF	7.146±0.675	3.861±0.349#	7.885±0.431*	7.457±0.713*	0.798±0.065*
ZR	3.046±0.283	2.363±0.343#	3.118±0.217	3.454±0.372*	1.783±0.150
Medulla	0.904±0.064	0.698±0.041#	0.768±0.051	0.915±0.043*	0.587±0.055
Volume of cells (μm^3)					
ZG	1474±145	1162±54#	1648±144*	1160±77	1158±59
ZF	2854±136	2087±112#	3224±197*	2600±204*	1586±150*
ZR	1278±79	1193±69	1634±82*	1486±115	3428±159*
Volume of nuclei (μm^3)					
ZG	243±10	222±22	251±12	260±8	239±18
ZF	257±16	214±26#	286±13*	349±14*	251±23
ZR	202±12	159±8#	199±6	260±20*	395±19*
Number of cells ($x10^6$)					
ZG	1650±173	1270±121#	1438±131	1566±93	726±37*
ZF	2734±182	1760±223#	2718±173*	2734±277*	500±66*
ZR	2227±57	1654±274#	1732±141	1892±210	471±46*

Abbreviations: DMSO, dimethylsulphoxide-vehicle; ZG, zona glomerulosa; ZF, zona fasciculata; ZR, zona reticularis. Values are given as mean for 5 animals in each group ± SEM. # $p<0.05$ *vs* Saline + DMSO and * $p<0.05$ *vs* Saline + EDS.

concentration was significantly elevated in the serum, but not in the adrenals of these animals.

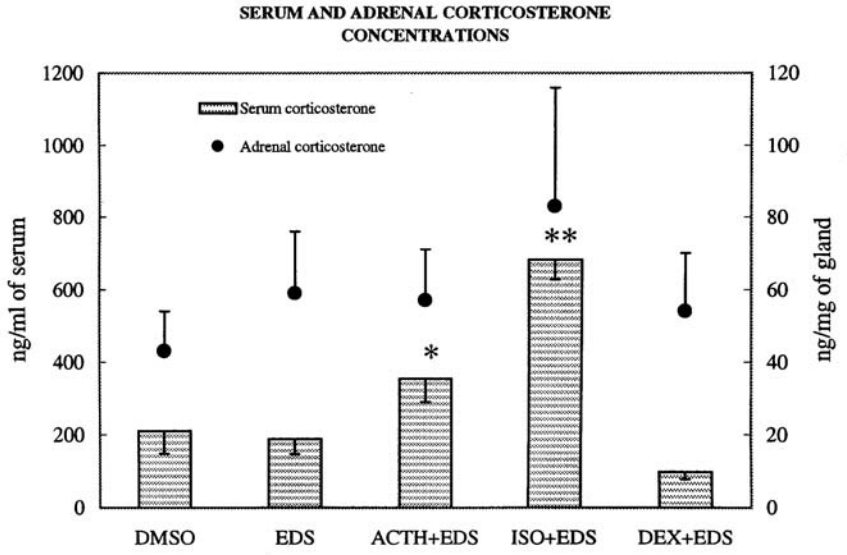

Figure 1. Serum (bars) and adrenal (dots) corticosterone concentrations in rats injected with saline and DMSO-vehicle, saline and EDS, and combinations of ACTH, isoproterenol (ISO) or dexamethasone (DEX) with EDS. Results are expressed as means ± SEM. The final number of corticosterone samples was 5-7 in the group. $^{*}p<0.05$ and $^{**}p<0.01$ *vs* the EDS-injected rats

Treatment with ISO and EDS enhanced the volume of the whole gland, ZF, ZR and the medulla. In the ZF increases were recorded in the cell and nuclear volume and the total cell number, whereas in the ZR only the mean nuclear volume was enlarged. In comparison with the values measured in rats injected with saline and EDS, serum corticosterone concentrations were almost 4 times higher. Adrenal corticosterone concentrations, however, were not significantly altered.

In the rats that received dexamethasone and EDS, significant decreases were found in the adrenal volume and the volumes of the ZG and ZF compared to saline and EDS-injected controls. In the ZG the total number of cells was decreased, whereas in the ZF both the cell volume and the total number of cells were decreased. However, in the ZR cellular and nuclear volumes were greatly enhanced, while the total cell number was reduced. No change in the adrenal corticosterone concentrations was detected, and the almost 50% decrease in serum corticosterone concentration was not statistically significant.

DISCUSSION

The results of this work demonstrate that the toxic effects of EDS on the rat adrenal cortex can be modified by treatments known to affect the hypothalamo-pituitary-adrenal axis, i.e. by ACTH, dexamethasone or isoproterenol, the β-adrenoceptor agonist.

As was shown earlier (Plećaš *et al*. 1997), adrenal gland weight is markedly decreased on day 15 after administration of a single dose of EDS and atrophic changes, measured stereologically, were most prominent in the ZF and ZR. However, the regressive changes in the gland were not accompanied by alterations in glandular and serum corticosterone levels. Several explanations for this discrepancy are possible. Firstly, the adrenal cortex has a tremendous functional reserve (Ribelin, 1984) and the magnitude of cortical atrophy evoked by EDS might be insufficient to be reflected by corticosterone levels. On the other hand, during the 15-day period after EDS administration the regenerative process might already have started, since adrenal weight is fully restored 30 days following injection (Plećaš *et al*. 2001). In almost all biological systems affected by EDS the nature of the changes has been found to be transitory; for instance, thymic weight recovers by 7 days after injection of EDS (Leeming *et al*., 1991). A compensatory stimulation of the inner zone might also occur. In favor of this latter assumption is the marked hypertrophy of a number of parenchymal cells in the ZR of EDS-injected rats observed in this and previous work (Plećaš *et al*. 1997).

Administration of ACTH or isoproterenol 4 days before and 5 days after EDS almost completely prevented adrenocortical regression.

Compared with EDS-injected animals, in rats injected with both ACTH and EDS, significant increases were found in the glandular and ZF and ZR volumes, the mean cellular volume in all cortical zones, as well as in the volume of nuclei and the total number of parenchymal cells in the ZF. Stereological parameters were similar to those in DMSO-injected controls except for the ZR cell volume, which was significantly greater (28%, $p<0.05$). The protective effect of ACTH against the toxic influence of EDS on the adrenal cortex is analogous to the action of human chorionic gonadotrophin on rat Leydig cells in the presence of EDS (Jackson and Jackson, 1984). The considerably elevated serum corticosterone level in these animals indicates that parenchymal cells preserve their functional capacity and respond to ACTH by corticosterone secretion. Whether the action of ACTH on adrenocortical cells exposed to EDS is direct or *via* endogenous glucocorticoids, as has been shown for testosterone and Leydig cells (Jackson and Jackson, 1984), remains to be established. Yet, it has to be pointed out that the synthetic glucocorticoid, dexamethasone, augmented the harmful effects of EDS on adrenocortical structure.

Isoproterenol, administered according to the same experimental protocol, had similar effects as ACTH, particularly on the ZF. All morphometrical parameters measured in this zone, as well as the serum corticosterone concentration, were significantly greater than in rats injected with saline and EDS. These results confirm our previous findings that a longer, 15-day, treatment with isoproterenol markedly stimulated corticosterone secretion both in intact and EDS-injected rats

(Plećaš *et al*., 1999). The site(s) of isoproterenol action is difficult to ascertain since β adrenoceptors are present in all components of the hypothalamo-pituitary-adrenal axis (Tilders *et al*., 1982; Shima *et al*., 1984; Sato *et al*., 1989; Nussdorfer, 1996; Ehrhart-Bornstein *et al*., 1998). Systemic administration of isoproterenol was shown to stimulate both ACTH and corticosterone secretion in the rat (Berkenbosch *et al*., 1981; Tilders *et al*., 1982), although this substance does not cross the blood-brain-barrier (Carlisle *et al*., 1999). Besides that, β adrenoceptor activation can influence the production of cytokines, particularly IL-6, by cortical macrophages (Maisel *et al*., 1989) or adrenal cells themselves (Judd *et al*., 1990; Judd and MacLeod, 1995). IL-6 alone and in synergism with ACTH stimulates the release of corticosterone from rat adrenocortical cells (Salas *et al*., 1990).

Isoproterenol, unlike ACTH, did not influence the morphometric characteristics of the ZG of rats injected with EDS. It is well documented that catecholamines, acting on β adrenoceptors, stimulate ZG cells either directly (Horiuchi *et al*., 1987) or through renin secretion (Holmer *et al*., 1997). We reported that chronic propranolol treatment decreased the volume of the ZG and the total number of parenchymal cells in it (Plećaš *et al*., 1996). It seems that in the presence of EDS the ZG cells lose their ability to respond to b adrenoceptor stimulation by significant morphological alteration. This finding may also indicate that different mechanisms are involved in the regulation of ZG cells by ACTH and isoproterenol when they are exposed to the toxic influence of EDS.

In rats whose adrenals were already suppressed with dexamethasone (Lecœniewska *et al*., 1992), the effects of EDS were much more pronounced. Compared with the values measured in saline and EDS-injected controls significant reductions were found in the volume and cellularity of all cortical zones and the mean volume of the ZF cells. However, despite severe adrenal atrophy an almost 50% decrease in mean serum corticosterone concentration was not statistically significant. Most probably, the compensatory hypertrophy of the ZR cells, which in rats produce corticosterone (Almeida *et al*., 1998), contributed to the maintenance of the blood corticosterone level.

Before making a conclusion, it is necessary to comment on the use of ether anaesthesia when it is known that ether stress elevates blood corticosterone level (Laczi *et al*., 1994). To avoid this rise, blood samples were taken within 2 minutes of total anaesthesia (Villas *et al*., 1991). Hence, the basal concentration of serum corticosterone of untreated animals, which were kept and handled in the same way as the experimental rats, was found to be 165.28±15.51 ng/ml. This concentration is in the range or even lower than some values obtained for control rat blood taken at the same time of day after decapitation (Rebuffat *et al*., 1989; Lecœnievska *et al*., 1992; Pignatelli *et al*., 1995).

The results of the morphometric study presented in this work demonstrate that stimulators of adrenocortical function and growth, such as ACTH or β adrenoceptor activation, protect rat adrenal cortex from the toxic effects of EDS, while dexamethasone, as a powerful inhibitor, potentiated those effects.

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UTICAJ ACTH, IZOPROTERENOLA I DEKSAMETAZONA NA ODGOVOR NADBUBREŽNE ŽLEZDE PACOVA NA ETANDIMETANSULFONAT (EDS): STEREOLOŠKO ISPITIVANJE

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SADRŽAJ

Alkilirajući agens etandimetansulfonat (EDS), pored toksičnog delovanja na Lajdigove ćelije semenika, izaziva izraženu atrofiju kore nadbubrežne žlezde odraslih pacova. U ovom radu je ispitivan uticaj devetodnevnog tretmana sa ACTH (40 IU/kg/d), izoproterenolom (120 μg/kg/d) ili deksametazonom (0.25 mg/kg/d) na morfološke promene kore nadbubrega koje izaziva jedna intraperitonealna injekcija EDS (75 mg/kg). Tretmani su započeli 4 dana pre davanja EDS i nastavljeni su još 5 dana, a životinje su žrtvovane 15 dana posle aplikacije EDS. U prisustvu ACTH ili izoproterenola ne ispoljavaju se promene u stereološkim parametrima kore nadbubrega koje izaziva EDS. Oba tretmana značajno povećavaju i koncentraciju kortikosterona u serumu. Deksametazon, međutim, pojačava toksične efekte EDS; zapremina svih zona kore i broj parenhimskih ćelija u zonama su smanjeni, ali preostale ćelije retikularne zone pokazuju značajnu hipertrofiju, koja je verovatno odgovorna za održavanje sekrecije kortikosterona kod ovih životinja. Rezultati rada pokazuju da se toksični efekti EDS na koru nadbubrega mogu sprečiti visokim nivoom ACTH ili stimulacijom β adrenalinskih receptora, a potencirati egzogenim glukokortikoidom.